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Merlin

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13. ABSTRACT (Maximum 200 Words) Neurofibromatosis type2 (NF2) is a familial cancer syndrome that features the development of nervous system tumors. The NF2-encoded protein, merlin, localizes to the membrane:cytoskeleton interface, raising the intriguing question of how a protein that occupies such a physical niche controls cell proliferation. To generate an animal model for NF2 and to build the foundation for delineating the molecular function of merlin, we established a Nf2-mutant mouse strain through genetic engineerins. Nf2 ^{+/-} mice develop a spectrum of tumors that is distinct from that of their human counterparts, including osteosarcomas and hepatocellular carcinomas, which exhibit loss of the wild-type Nf2 allele. Embryos that are homozygous for a null Nf2 mutation fail to gastrulate, while chimeric embryos partially composed of Nf2 ^{-/-} cells develop additional defects, including during cardiac development. Together, these observations indicate a requirement for merlin function in several different cell types in the mouse; the study of Nf2 function in these cell types formed the basis of this proposal. The following report describes our accumulating data that indicates an important and general role for Nf2 function in cell differentiation. In addition, data from these and other studies in our laboratory point to a general role for merlin function in plasma membrane organization in different cell types.				
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Table of Contents:

Front page	1
Standard Form 298	2
Table of Contents	3
Introduction	4
Body	5
Key Research Accomplishments	9
Reportable Outcomes	10
Conclusions	11
References	12
Appendices	13-18

Introduction:

Mutations in the *NF2* tumor suppressor gene underlie the familial cancer syndrome neurofibromatosis type 2 (NF2). Human NF2 patients are predisposed to developing schwannomas and meningiomas of the central nervous system. The *NF2*-encoded protein, merlin, localizes to the membrane:cytoskeleton interface, thus occupying an intriguing physical niche for a growth suppressing protein. We have generated a *Nf2*-mutant mouse strain and thus the foundation for defining the molecular consequences of *Nf2* loss (McClatchey et al., 1997). We found that, like their human counterparts, *Nf2*^{+/-} mice are predisposed to tumor development. However, instead of schwannomas, *Nf2*^{+/-} mice primarily develop osteosarcomas, and, with lower frequency, hepatocellular carcinomas and fibrosarcomas, all of which exhibit loss of the wild-type *Nf2* allele. Embryos that are homozygous for a null *Nf2* mutation fail to gastrulate, due to a defect in the extraembryonic lineage (McClatchey et al., 1997). Furthermore, chimeric embryos partially composed of *Nf2*-deficient cells develop several additional developmental defects, including during cardiac development (A.I.M., unpublished). Taken together, these observations indicate a requirement for merlin function in several different cell types in the mouse. The availability and manipulability of both wild-type and *Nf2*^{-/-} primary murine cells of these types make them attractive tools for delineating the molecular function of merlin. Thus the study of *Nf2* function in several different cell types formed the basis of this proposal. In particular, we proposed an investigation of merlin function in cardiomyocytes during embryonic development and ES cell differentiation, and in osteoblasts and hepatocytes, from which murine *Nf2*^{-/-} tumors derive.

We submitted a progress report one year ago that described significant progress in studying the requirement for merlin function in cardiomyocyte differentiation by establishing an in vitro differentiation system for ES cells. We also described studies aimed at detecting changes in merlin levels and/or phosphorylation in Hepa1-6 cells (an immortalized murine hepatoma cell line) under various conditions in culture and in mouse liver tissue upon regeneration after partial hepatectomy. Finally, we established a system for in vivo introduction of gene expression into the liver using GFP expression as proof of principle. Following is an update of progress made since that time.

Body:

Nf2 function in ES cell differentiation

Although *Nf2*^{-/-} embryos fail to gastrulate, the defect is an extraembryonic one, and thus is easily rescued in a chimeric setting (McClatchey et al., 1997). Chimeric embryos partially composed of *lacZ*⁺; *Nf2*^{-/-} cells develop several defects, including tumor-like lesions in the myocardium. *Nf2*-deficiency is sufficient for their formation as all *lacZ*⁺ cells in the myocardium form such lesions. Cardiac lesions can be detected early during heart morphogenesis (ie E9.0), suggesting that they may arise via failed differentiation. Embryonic stem (ES) cells can differentiate into a number of cell lineages in vitro, including cardiomyocytes, which are easily recognized by their ability to physically beat in a culture dish. In progress described last year, we monitored individual ES-derived embryoid bodies (EBs), and found that while up to 60% of wild-type EBs contain beating foci, none of the *Nf2*^{-/-} EBs ever contained beating foci. This difference was not obviously due to a global proliferative defect in *Nf2*^{-/-} EBs, as hematoxylin and eosin stained paraffin sections of wild-type and *Nf2*^{-/-} EBs did not reveal gross morphological differences between the two at early stages of differentiation. Moreover, expression of the early primitive streak and cardiac/neural crest markers *brachyury* and the *mef2C*, were detected in both wild-type and *Nf2*^{-/-} EBs. However, it is obvious that at later stages of development, wt EBs exhibit a considerably more complicated pattern of differentiation, based upon histological and microscopic analyses. Similarly, teratomas that develop subcutaneously from *Nf2*^{-/-} ES cells are composed of several highly differentiated cell types, indicating that *Nf2*^{-/-} ES cells are capable of specialized differentiation. However, *Nf2*^{-/-} teratomas exhibit a unique, but restricted spectrum of cell types, being composed largely of primitive neuroepithelium, keratinocytes and melanocytes, none of which are common components of teratomas derived from wild-type cells. Together these studies indicate that merlin may play an important role in establishing a program of cell differentiation and indicate that we should continue to explore the broad differentiative capabilities of *Nf2*^{-/-} ES cells..

This past year we have expanded these studies in several different ways: First, we have carried out experiments designed to address whether the failure of *Nf2*^{-/-} cells to become beating cardiomyocytes was cell-autonomous or non-autonomous. To this end we generated EBs composed of various ratios of wild-type and *Nf2*^{-/-} ES cells (all wt, 10:1 wt:*Nf2*^{-/-}, 1:1 wt:*Nf2*^{-/-}, 1:10 wt:*Nf2*^{-/-}, all *Nf2*^{-/-}). We found that the percentage of beating EBs in this experiment roughly correlated with the percentage of wild-type cells present, suggesting but not proving a cell-autonomous requirement for *Nf2* function in cardiomyocyte differentiation in vitro. Moreover, at late stages of EB development, a clear correlation between increased histological complexity and wild-type cell contribution is observed (Figure 1). LacZ and H&E staining of paraffin sections of these EBs confirms the roughly appropriate ratios of wt and *Nf2*^{-/-} contribution.

Our studies of the differentiative capacity of *Nf2*^{-/-} ES cells indicates that they are capable of complex programs of differentiation, yet exhibit a markedly different program of differentiation relative to wild-type. In order to continue to broadly explore the differentiative capacity of *Nf2*^{-/-}

ES cells, we have evaluated the expression of additional markers in our *Nf2*^{-/-} EBs. Notably, consistent with the ability of *Nf2*^{-/-} EBs to express brachyury, they also express bone morphogenic protein receptors I-A and I-B (BMPRI-A, BMPRI-B) additional mesodermal markers. *Nf2*^{-/-} EBs also express c-kit ligand, but not embryonic globin (β H-1), both markers of primitive hematopoiesis, perhaps indicating a specific block in the hematopoietic lineage (Figure 2). Many additional markers such as the c-kit receptor, BMP4, flk-1, GATA-1 and -2 are currently being tested.

Protocols for the preferential differentiation of ES cells into specific lineages are becoming increasingly available. For example, conditions for the enrichment of hematopoietic cells, osteoblasts, melanocytes and neuronal precursors have been recently described. To complement the broad marker analysis described above, we will explore their ability to perform in a number of these protocols. Teratomas derived from *Nf2*^{-/-} ES cells exhibit a unique pattern of differentiation, including keratinocytes, melanocytes and what appears to be primitive neuroepithelium. By immunohistochemical analysis, we found relatively high levels of expression of nestin, a marker of neuronal precursors in these tumors, confirming that they contain primitive neuroepithelium (not shown). Therefore we have begun to explore the ability of *Nf2*^{-/-} ES cells to become neuronal precursors in vitro. Our preliminary findings are that *Nf2*^{-/-} ES cells can become nestin positive precursors in vitro, albeit at reduced numbers relative to wild-type. However, unlike wild-type cells, *Nf2*^{-/-} cells cannot differentiate fully into mature, β 3-tubulin-positive neurons (data not shown). Taken together, these results point to a critical role for NF2 function in programming the differentiation of several different lineages to maturity (also see below).

***Nf2* function in osteoblast differentiation**

Nf2^{+/-} mice predominantly develop osteosarcomas of the spine and cranium; a subset of *p53*^{+/-} mice also develops osteosarcomas. In contrast to humans, the *Nf2* and *p53* loci reside on the same mouse chromosome. Therefore we generated *Nf2*^{+/-};*p53*^{+/-} cis and trans mice and asked not only whether cooperativity between the two mutations would be evident, but whether linkage of the two cancer predisposing mutations affected the tumor phenotype. We found that mice carrying compound mutations in both the *Nf2* and *p53* tumor suppressor loci exhibit markedly reduced latency of osteosarcoma development particularly when the mutations are in cis. Thus *Nf2* function is of obvious importance in the osteoblast and we are very interested in exploring the molecular basis of merlin function in this system. In progress described last year, we had established a large panel of osteosarcoma cell lines of various genotypes for study and we had begun to examine the pattern of osteogenic markers that they express.

During the past year we have devoted considerable effort to the generation and study of a panel of primary osteoblast cultures of various genotypes including wild-type, *Nf2*^{+/-}, *p53*^{+/-} and *Nf2*^{+/-};*p53*^{+/-} cis, as well as immortalized *Nf2*^{-/-};*p53*^{-/-} and *p53*^{-/-} osteoblast cell lines. Most recently we have used *Nf2*^{flox/flox} mice kindly provided by Marco Giovannini to generate *Nf2*^{-/-} osteoblasts. First we examined the levels and phosphorylation of merlin in the primary cultures under various conditions (Figure 3). We found that the ratio of hypo- to hyper-phosphorylated forms of merlin increased with increasing osteoblast density as we reported in fibroblasts (compare lanes marked 'S' for subconfluent with 'C' for confluent; Shaw et al., 1998). We also found a dramatic

decrease in the total levels of merlin upon differentiation (lanes marked 'D'). Differentiated cultures produced relatively large amounts of calcified bone and exhibited expression of osteogenic markers such as osteocalcin (data not shown). We also found that *Nf2*^{+/-} osteoblasts expressed reduced levels of merlin under all conditions such that merlin levels are barely detectable upon differentiation. In other studies ongoing in the laboratory, we have found that *Nf2*^{-/-} fibroblasts exhibit several growth advantages in culture, including loss of contact inhibition; thus *Nf2*^{-/-} fibroblasts never reach saturation density and form a multilayer (in preparation). We examined the growth properties of *Nf2*^{+/-} and *Nf2*^{+/-};*p53*^{+/-} cis and *p53*^{+/-} osteoblasts and found that *Nf2*^{+/-} and particularly *Nf2*^{+/-};*p53*^{+/-} cis osteoblasts grow to a higher density in culture and produce more calcified bone than wild-type cultures (data not shown). Interestingly, high cell density is known to be required for and to stimulate osteoblast differentiation.

Next we asked whether we could recapitulate the genetic basis of tumorigenesis in *Nf2*^{+/-};*p53*^{+/-} cis mice in vitro. *Nf2*^{+/-};*p53*^{+/-} cis mice develop multiple osteosarcomas and survive only to about 5 months of age (McClatchey et al., 1998). Tumors from these mice exhibit loss of both the wild-type *Nf2* and *p53* alleles and, where informative, for polymorphic markers in between, suggesting that the entire wild-type chromosome had been lost (Figure 4B). In other studies we have found that *Nf2*^{+/-};*p53*^{+/-} cis MEFs readily lose the wild-type chromosome by passage 5-6 (Figure 4A), thus becoming an established immortalized cell line. Therefore we asked whether this strategy could be applied to osteoblasts. We readily achieved loss of the wild-type chromosome upon passage *Nf2*^{+/-};*p53*^{+/-} cis and *p53*^{+/-} osteoblasts and generated immortalized *Nf2*^{-/-};*p53*^{-/-} or *p53*^{-/-} osteoblast lines (Figure 4C). A comparison of the capacity of *Nf2*^{+/-};*p53*^{+/-} cis, *p53*^{-/-} and *Nf2*^{-/-};*p53*^{-/-} osteoblasts to differentiate in culture revealed that whereas both the *Nf2*^{+/-};*p53*^{+/-} and *p53*^{-/-} cells produced copious amounts of calcified bone upon differentiation, the *Nf2*^{-/-};*p53*^{-/-} cells did not produce visible calcified bone (Figure 5A). As this could either be due to the fact the *Nf2*^{-/-};*p53*^{-/-} osteoblasts produce extracellular matrix but cannot elaborate it or fail to turn on the differentiation program from an early stage. Therefore we examined the mRNA levels of the early osteogenic marker osteocalcin (OC) in these cultures by Northern blot analysis and found that in contrast to wild-type, *Nf2*^{+/-}, *p53*^{+/-}, *Nf2*^{+/-};*p53*^{+/-} cis and *p53*^{-/-} cultures, *Nf2*^{-/-};*p53*^{-/-} cells did not turn on osteocalcin (Figure 5B). These data suggest that loss of *Nf2* specifically leads to a failure of osteoblast differentiation. We are currently examining the ability of *Nf2*^{-/-};*p53*⁺ osteoblasts to differentiate. To see whether *Nf2*-deficient osteosarcomas similarly show reduced levels of osteocalcin, we examined osteocalcin expression in a panel of *Nf2*-expressing, *p53*-deficient osteosarcoma lines derived from *p53*^{+/-} mice versus *Nf2*-deficient osteosarcoma cell lines and found that osteocalcin mRNA levels were markedly higher in the *p53*-deficient, *Nf2*-expressing cell lines (Figure 6).

The kinetics of tumor development in *Nf2*^{+/-};*p53*^{+/-} cis mice suggests that other mutational events may not be necessary for tumor formation in these mice. Therefore we tested the ability of our immortalized *Nf2*^{-/-};*p53*^{-/-} osteoblasts to form tumors when injected subcutaneously or metastases when injected into the tail vein of nude mice. Strikingly, in a pilot experiment, one of these cell lines was able to form both tumors and metastases. Studies currently underway aim to examine the ability of a larger panel of primary and immortalized osteoblast lines to form tumors or metastases in these assays. These studies suggest that merlin may be required for osteogenic differentiation and imply that the failure to differentiate leads to hyperproliferation and tumor

formation. Together with the ES cell differentiation studies described above, our data indicate a critical requirement for merlin in differentiation of many cell types.

***Nf2* function in hepatocytes**

As we reported last year, our preliminary studies of merlin regulation in Hepa1-6 cells and in the regenerating liver were disappointing. We could not detect changes in merlin phosphorylation or levels in cultured Hepa1-6 cells under a variety of conditions or in liver tissue undergoing regeneration after partial hepatectomy. One possible explanation for this was that perhaps only a small intracellular pool of merlin was regulated by these conditions. Ongoing studies in our laboratory seek to define merlin intracellular localization and movement in fibroblasts by cell fractionation. In those studies we have identified a distinct pool of merlin that is localized to the membrane and is preferentially hyperphosphorylated. A second pool is Triton X-100 insoluble (presumably cytoskeleton-associated) and preferentially hypophosphorylated. These studies challenge the accepted view that merlin, like the ERMs, transitions between cytosolic and membrane:cytoskeleton linking states. Using these fibroblast studies as a guide, we asked whether the same compartmentalization of merlin was found in liver cells. Figure 6A shows that, indeed, Hepa1-6 cells exhibit the same localization trend. Interestingly, Hepa1-6 cells have a considerably higher ratio of hypo- to hyper-phosphorylated merlin than fibroblasts. Accordingly, the insoluble fraction in these cells contains almost entirely hypophosphorylated merlin. Moreover, upon subfractionation of fibroblast membranes by several methods, we have found that merlin is present in caveolin-containing lipid rafts (data not shown). Thus we examined the localization of merlin to Triton-insoluble rafts in Hepa1-6 cells and in membranes extracted from adult liver tissue. Figure 6B shows that not only does merlin localize to Triton-insoluble membrane fractions, but EGF treatment of confluent, starved Hepa1-6 cells results in a dramatic reduction in the levels of merlin in this membrane compartment. These results suggest that merlin may function to control signaling via membrane compartmentalization. We have recently described a relationship between merlin and the small GTPase Rac (Shaw et al., 2001). Activation of Rac leads to phosphorylation and increased solubility of merlin; conversely, merlin can negatively regulate Rac signaling. Again, using our fibroblast studies as a guide, we will continue to explore the importance of merlin localization to specific membrane compartments in liver cells. Through the generation of *Nf2*^{-/-} hepatocytes, we will examine the dependency of other signaling molecules upon merlin localization to specific membrane compartments in the liver.

To study the requirement for *Nf2* function in the liver vivo we reported progress last year in establishing a system for in vivo transfection and showed proof of principle evidence of GFP expression upon transfection of a GFP-expression plasmid into the liver via injection into the tail vein. We have now used this system to transfect the Cre recombinase into *Nf2*^{lox/lox} mice that we recently obtained; both short and long-term analyses are underway. In addition, to remove *Nf2* throughout the liver, we have obtained albumin-Cre transgenic mice, which we are crossing to *Nf2*^{lox/lox} mice in order to achieve complete organ-specific removal of *Nf2* gene expression in the liver.

Key Research Accomplishments:

- *Nf2*^{-/-} ES cell derived embryoid bodies and teratomas contain highly differentiated cell types, but the spectrum of cell types that they exhibit is remarkably different from those derived from wild-type ES cells, suggesting that *Nf2*-deficiency alters the differentiation program of these cells.
- In vitro analysis of primary osteoblast cultures of a number of different genotypes has been established. Preliminary results indicate that *Nf2*-deficiency prevents osteogenic differentiation. Moreover, *Nf2*^{+/-};p53^{+/-} cis osteoblasts are growth advantaged and readily immortalized in vitro. Remarkably, although loss of the wild-type chromosome appears to be their only genetic alteration, these cells are tumorigenic and metastatic but cannot initiate an osteogenic program. This provides an exciting system for studying the requirements for *Nf2* function in growth suppression and differentiation in vitro that will parallel our in vivo analysis of *Nf2*-mutant mice.
- Subcellular fractionation of Hepa1-6 cells reveals that merlin levels and phosphorylation are compartmentalized in a manner that is analogous to our studies of fibroblasts. These studies identify the existence of distinct membrane and cytoskeletal pools of merlin. Moreover, in both fibroblasts and hepatocytes, merlin can be detected in triton-insoluble lipid rafts. In hepatocytes, EGF treatment results in a dramatic decrease in the levels of merlin that are detectable in rafts. Using our fibroblast studies as a guide, we will continue to explore merlin subcellular localization and movement in liver cells as well as the dependency of membrane initiated signaling upon merlin function.
- Using the relatively efficient and inexpensive system for in vivo gene transfer into the liver that we developed and reported last year, we have introduced Cre expression into some cells in the mature liver for both short and long term analyses. We have also begun crossing *Nf2*^{flox/flox} mice with Cre-albumin transgenic mice in order to completely remove *Nf2* expression from the liver in vivo.

Reportable outcomes:

Some of this data has been incorporated into seminar presentations given by Dr. McClatchey at the following meetings:

January, 2001

Invited seminar: "Cancer and the Cell Cycle", Swiss Institute for Experimental Research (ISREC), Lausanne, Switz.

February, 2001

Invited seminar, Columbia University, NY

April, 2001

Invited seminar: Duke University, Durham, NC

May, 2001

Invited seminar: National Neurofibromatosis Foundation Annual Meeting, Aspen, Colorado

June, 2001

Invited seminar: MD Anderson Cancer Center, Houston TX

October, 2001

Invited seminar: "Cell Cycle Meeting", Colrain, MA

A manuscript describing the requirement for *Nf2* function in osteogenic differentiation is in preparation. We expect to submit it by the end of the year.

Conclusions:

We have made considerable progress in our efforts to study merlin function in several different cell types. Our studies of *Nf2*^{-/-} ES cell differentiation suggest that *Nf2*^{-/-} cells are capable of differentiating into mature cell types but that the program of differentiation carried out by *Nf2*^{-/-} cells is altered. Over the next year we will amass a large amount of data concerning the expression of a large panel of markers in these cells in an effort to better understand the nature of their altered differentiation program. We are also currently generating additional chimeric embryos (through a blastocyst injection facility here at MGH) so that we can examine the expression of many of these markers in vivo in chimeric embryos. Through these studies we will also focus on the cardiac lineage and the interesting cardiac defect that we have characterized in chimeric embryos. However, our longterm goal is to utilize all of this information to better understand the molecular consequences of *Nf2* loss in tumorigenesis. As our studies imply that failed differentiation may be a general mechanism whereby loss of *Nf2* promotes tumorigenesis, we will use all of the tools available to us to delineate that mechanism.

We are excited by the results of our osteoblast studies and the indication that *Nf2* plays an important role in osteogenic differentiation as well. This line of investigation therefore nicely dovetails with the ES cell differentiation studies described above and point to a mechanism whereby *Nf2* loss leads to tumorigenesis. A relationship between cell differentiation and tumorigenesis has long been postulated, but the nature of this relationship remains obscure. It has recently been determined that the Rb tumor suppressor physically interacts with and promotes the activity of the osteoblast-specific transcription CBFA1 (Thomas et al., 2001). When Rb function is lost, osteogenic differentiation is impaired; familial retinoblastoma patients have a dramatically increased risk of developing osteosarcoma. There are obvious parallels between these data and ours. As the investigators who carried out this study are here at Harvard, we have communicated with them and agreed to collaborate to further explore the parallels between our systems. Finally, our data have important implications for the general study of bone biology, which is a large human health issue.

The identification of a regulated pool of membrane-localized merlin affords exciting possibilities for defining the general mechanism of merlin function. Although the pilot studies that led to this observation are being carried out outside of the specific goals of this proposal, we have the unique opportunity to explore their applicability to other cell types that are clearly affected by *Nf2* loss through these studies. In particular, the liver is a uniquely manipulable organ affording the opportunity for careful in vivo study. In addition, hepatocytes are epithelial cells, providing the opportunity to study merlin function in polarized cells.

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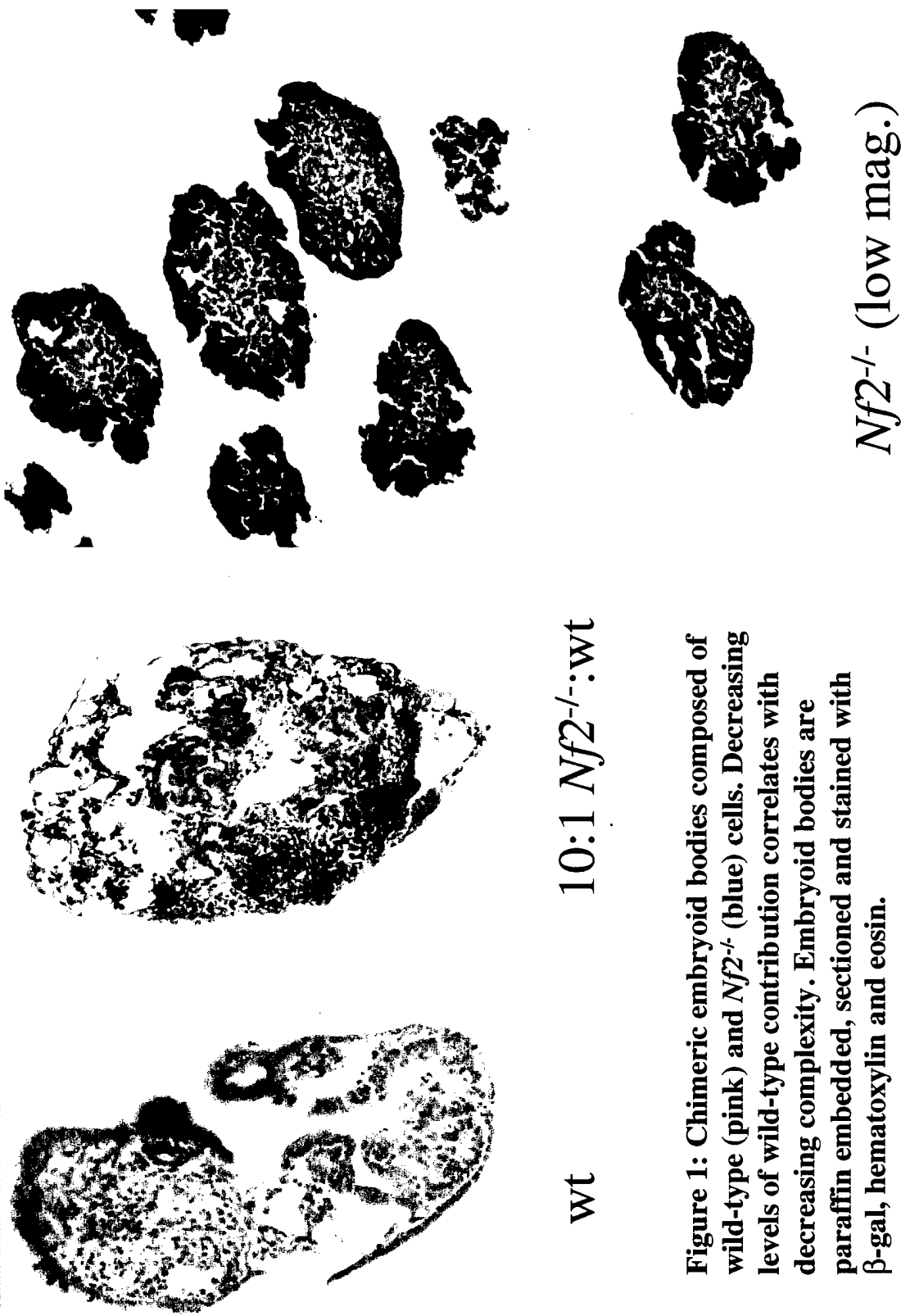


Figure 1: Chimeric embryoid bodies composed of wild-type (pink) and *Nf2*^{-/-} (blue) cells. Decreasing levels of wild-type contribution correlates with decreasing complexity. Embryoid bodies are paraffin embedded, sectioned and stained with β -gal, hematoxylin and eosin.

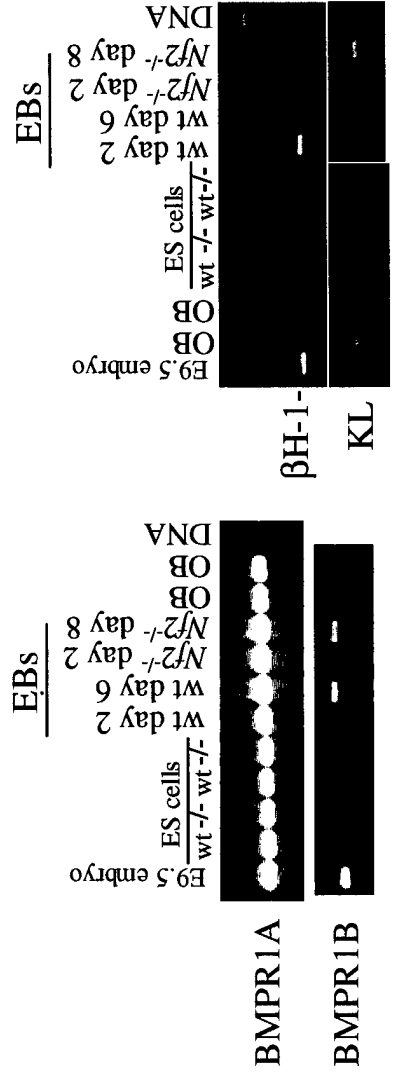


Figure 2: BMP receptor 1A and 1B (BMPR1A, BMPR1B), fetal globin (β H-1) and c-kit ligand (KL) expression in wild-type and *Nf2* $^{-/-}$ embryoid bodies (EBs).

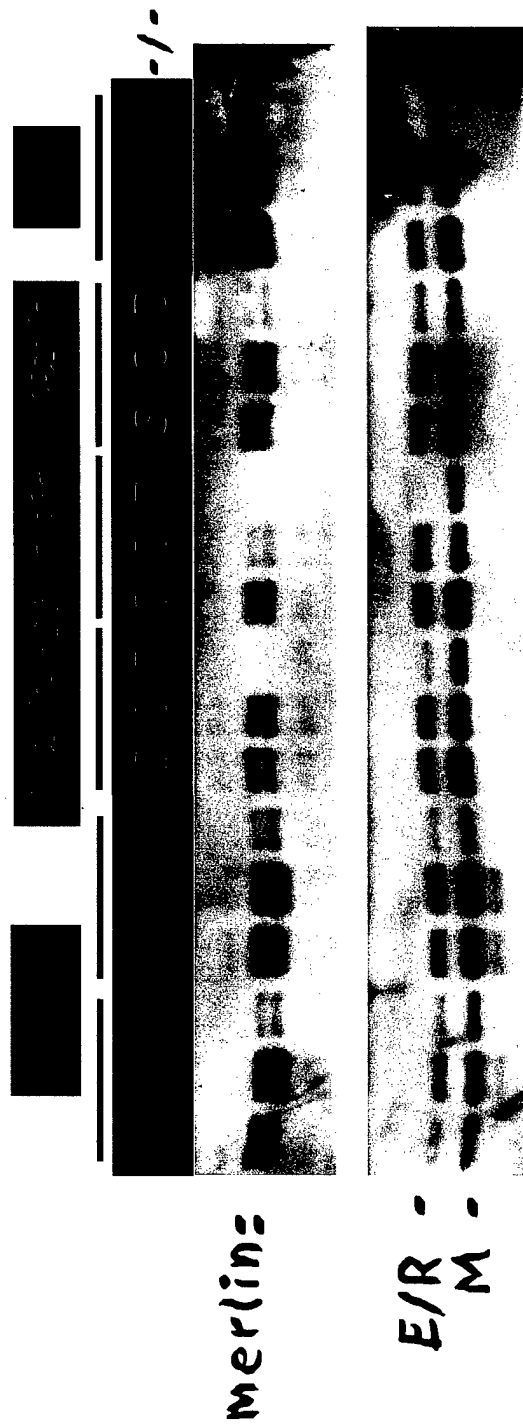
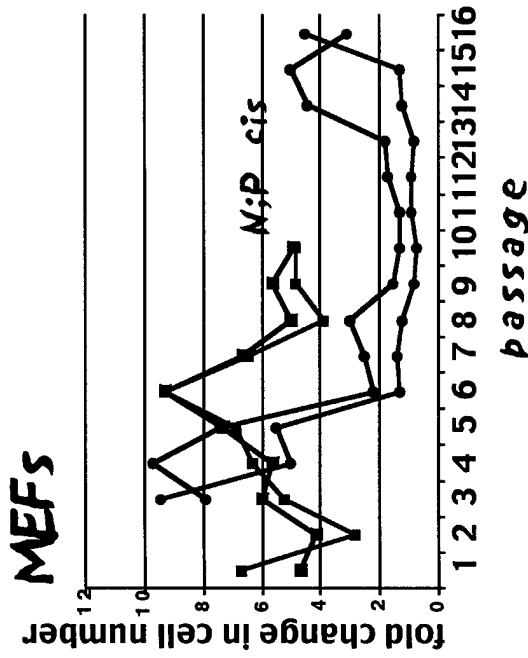
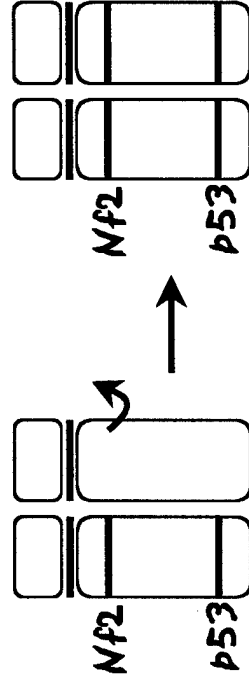


Figure 3: Merlin levels and phosphorylation in primary osteoblasts. The ratio of hypo- to hyperphosphorylated merlin increases upon confluence (C). In addition, the total levels of merlin decrease upon differentiation (D) in contrast to that of the ERM proteins. Merlin levels are approximately 50% in *Nf2^{+/-}* cells.

A.



B.



C. osteoblasts $Nf2^{+/-}; p53^{+/-}$ cis

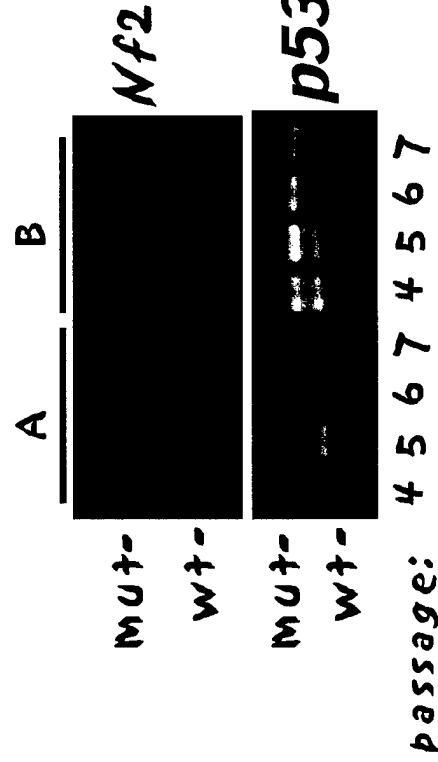


Figure 4: (A) Primary $Nf2^{+/-}; p53^{+/-}$ cis MEFs

readily undergo immortalization upon passage.

Immortalized lines are established by passage 9.

(B) These cells display loss of the wild-type $Nf2$ and

$p53$ alleles. (C) $Nf2^{+/-}; p53^{+/-}$ and $p53^{+/-}$ osteoblasts also

readily immortalize and lost the wild type $Nf2$ and

$p53$ alleles.

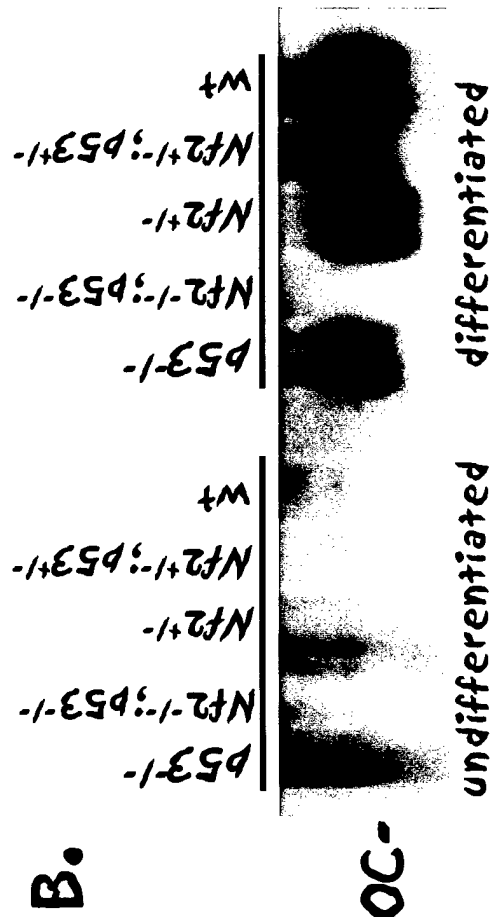
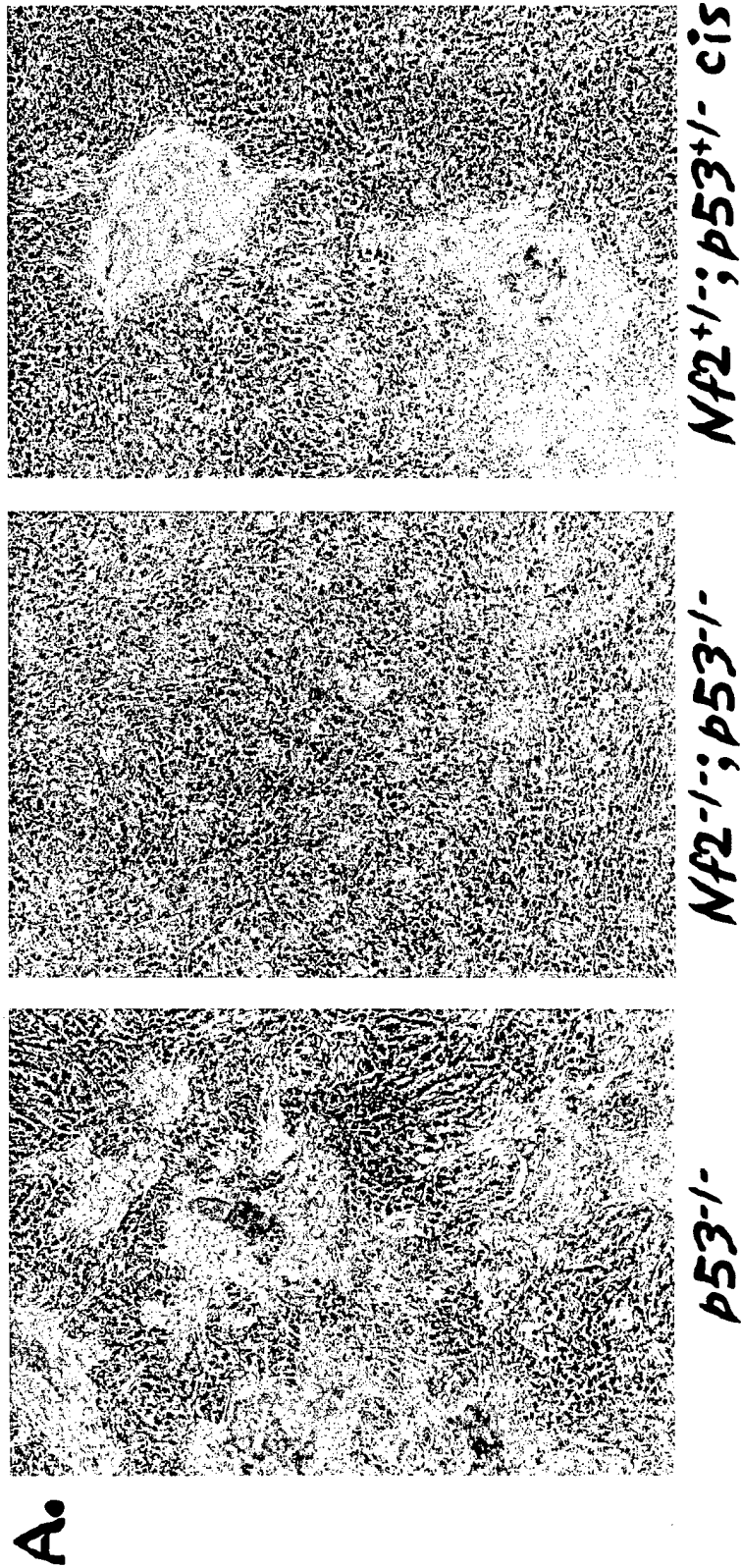


Figure 5: Immortalized *Nf2^{-/-}; p53^{-/-}* osteoblasts do not differentiate in vitro. (A) Phase contrast microscopy reveals abundant calcified extracellular matrix in *Nf2^{+/-}; p53^{+/-} cis* and *p53^{-/-}* but not *Nf2^{-/-}; p53^{-/-}* osteoblast cultures. (B) Northern blot analysis reveals that the early osteogenic marker osteocalcin (OC) is not expressed in *Nf2^{-/-}; p53^{-/-}* cells.

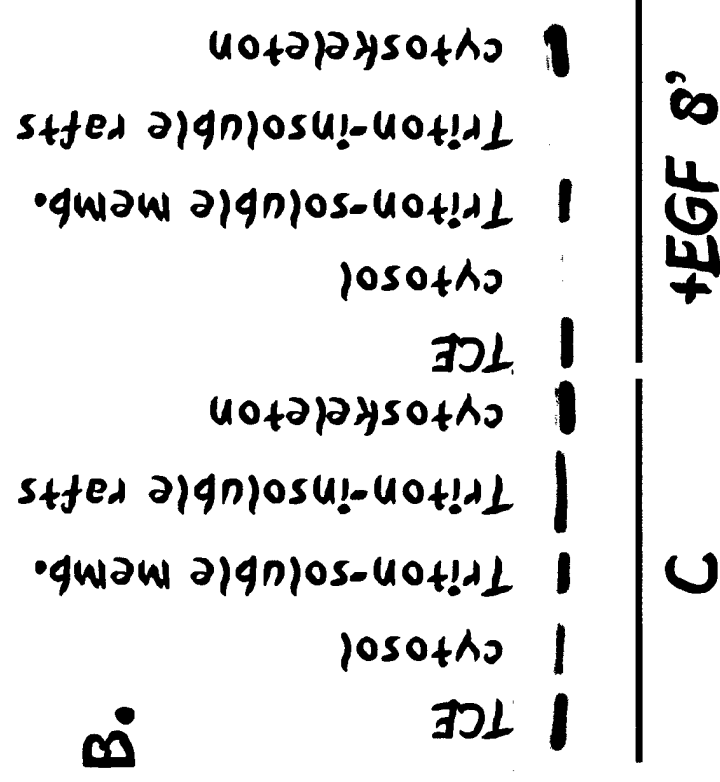
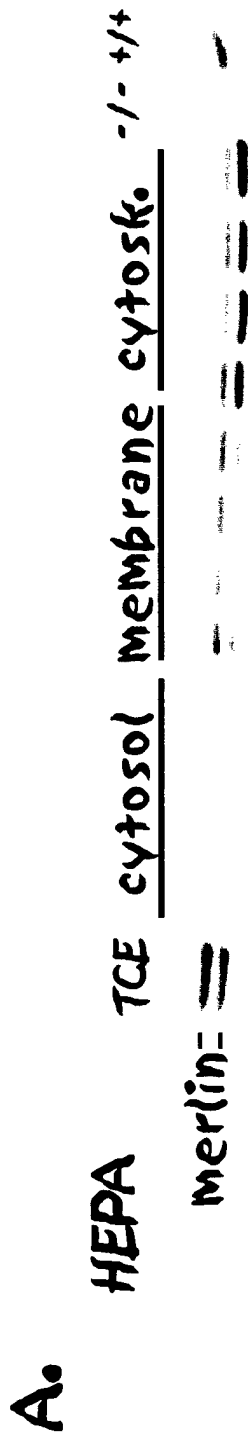


Figure 6: A membrane-localized pool of merlin in liver cells. (A) Isolation of Hepa1-6 cell membranes by high speed centrifugation reveals the presence of a pool of merlin that is enriched for the hyperphosphorylated form. In contrast, the insoluble, presumably cytoskeleton-associated pool is enriched with the hyperphosphorylated form of merlin. (B) Further fractionation of Hepa1-6 cells reveals that merlin localization to lipid rafts is drastically reduced upon EGF stimulation (8 mins).